## REMARKS

This Response is filed in connection with the Office Action mailed April 7, 2005. Claims 1 to 86 are pending. Claims 1 to 30 stand withdrawn from consideration as directed to non-elected subject matter. Claims 1 to 30, 32, 33, 39, 44, 50, 53, 74, 75, 77, 81 and 84 have been cancelled herein without prejudice. Applicants maintain the right to prosecute the cancelled claims in any related application claiming the benefit of priority of the subject application. New claims 87 to 113, based upon originally filed and elected claims 31 to 55, have been added. Accordingly, upon entry of the Response, claims 31, 34 to 36, 38, 40, 43, 47 to 49, 51, 52, 54, 55, 71 to 73, 76, 78 to 80, 82, 83 and 85 to 113 are under consideration.

*Regarding the Claim Amendments*

The amendments to the claims are supported throughout the specification or were made to address an informality. In particular, the amendment reciting "diabetes" is supported, for example, by claim 33, as originally filed. The amendment reciting "endocrine" cells is supported, for example, by claim 50, as originally filed. The amendment reciting "glucose-dependent insulinotropic polypeptide (GIP) promoter" is supported, for example, by claim 44, as originally filed. The amendment reciting "chromogranin A" promoter is supported, for example, at page 15, Table 1. The amendment reciting to "decrease blood glucose" is supported, for example, at page 32, lines 21-25, which discloses, *inter alia*, that a mucosal cell that produces insulin or a functional subsequence of insulin is useful for decreasing glucose (see, also, page 35, lines 20-22). The amendment reciting that "the endocrine cell transformation occurs *in vivo* via intra-cavity delivery" is supported, for example, at page 37, lines 19-23, which discloses *in vivo* delivery in order to produce encoded protein in the subject; and at page 39, lines 19-22, which discloses administration by routes including, for example, intra-cavity. The amendment reciting "type 1" diabetes and the amendment reciting "insulin-independent (type 2) diabetes" is supported, for example, at page 32, lines 11-14, which discloses disorders treatable by a method of the invention including, for example, insulin-dependent (type 1) and insulin-independent (type 2) diabetes. The amendment reciting "L-cell, S-cell, G-cell, D-cell, I-cell, Mo-cell, GR cell or entero-endocrine cell" is supported, for example, at page 25, lines 6-7 (see, also, page 28, lines 21-25). The amendment reciting "a progeny" of a stem cell, "a pluripotent progenitor cell or a multipotent progenitor cell" is supported, for example, at page 10, lines 27-28; and at

page 25, lines 3-6. The amendment reciting "undesirable body mass or obesity" is supported, for example, at page 33, lines 21-25. The amendment reciting that the undesirable body mass or obesity is reduced is supported, for example, at page 35, lines 26-30. Various amendments reciting tissue endocrine" and "mucosal tissue" were made in order to provide greater antecedent basis for the recited "mucosal tissue endocrine cells." Thus, as the claim amendments are supported by the specification or were made to address an informality, no new matter has been added. Accordingly, entry of the amendments is respectfully requested.

*Regarding the New Claims*

New claims 87 to 113 include the subject matter of originally filed claims 31 to 55 prior to entry of the amendments set forth herein. Claims 87 to 113 are therefore supported by originally filed claims 31 to 55. Claims 87 and 88 are also supported, for example, at page 25, lines 25-28; and at page 42, lines 25-27, which discloses, *inter alia*, endoscopes, feeding tubes, cannulas, intubation tubes and catheters for delivery to the gut. Claims 89 to 113 are also supported, for example, as set forth above for the claims amendments and at page 28, lines 14-18, which discloses introducing a protein into a subject to achieve therapy by transfecting a polynucleotide, including an expression control element in operable linkage with a nucleic acid encoding a protein into cultured mucosal cells, followed by implanting the transformed cells or progeny into the subject (see, also, page 29, lines 4-14). Thus, claims 87 to 113 are supported by the specification and no new matter has been added. Accordingly, entry of the new claims is respectfully requested.

## I. REJECTIONS UNDER 35 U.S.C. §112

The rejection of claims 31 to 36, 38 to 40, 43, 44 and 47 to 55 under 35 U.S.C. §112, first paragraph, as allegedly lacking an adequate written description, is respectfully traversed. Allegedly, "the structure of each of these genera could not be distinguished from each other." [Office Action, pages 4-5]

The specification adequately describes claims 31 to 36, 38 to 40, 43, 44 and 47 to 55. Applicants wish to remind the Patent Office that the specification need only apprise the skilled artisan of the invention in sufficient detail to demonstrate Applicants had possession of the invention in order to satisfy the written description requirement under 35 U.S.C. §112, first

paragraph. An adequate written description does not require a disclosure of every species encompassed by the claims, even in an unpredictable art. *In re Angstadt*, 537 F.2d 498, 502-503 (CCPA 1976); *Utter v. Hiraga*, 845 F.2d 993, 998-99 (Fed. Cir. 1988)). Thus, a description of every nutrient (e.g., sugar, carbohydrate, starch, polypeptide, amino acid or fat) that induces production of a protein is not required.

Here, structural features that characterize nutrients, including sugars, carbohydrates, starches, polypeptides, amino acids and fats are well known to the skilled artisan. For example, the skilled artisan knows that sugars are molecules that have a generic formula of $C_nH_{2n}O_n$ and are polyhydroxy compounds containing either a ketose of aldehyde group. Carbohydrates include sugars, starches and cellulose. The skilled artisan knows that carbohydrates that are simple sugars are monosaccharides and that simple sugars can combine to form polymers, or more complex carbohydrates. The skilled artisan knows that a combination of two simple sugars is a disaccharide. The skilled artisan also knows that proteins contain amino acids which are characterized by the $-CH(NH_2)COOH$ substructure. The skilled artisan knows that nitrogen and two hydrogens comprise the amino group, $-NH_2$, the acid entity is the carboxyl group, -COOH, and when the carboxyl group of one amino acid reacts with the amino group of another amino acid, a peptide bond -C(=O)NH- is created releasing a molecule of water ($H_2O$). The skilled artisan knows that peptides, polypeptides and proteins are polymers of amino acids. The skilled artisan further knows that fats, also known as triglycerides are hydrophobic molecules made from a combination of one molecule of glycerol with three fatty acids. The skilled artisan knows that fats can be either saturated or unsaturated depending on the structure of the long carbon-carbon chains in the fatty acids, that fats that contain no double bonds in the fatty acid chains are saturated fats, and that unsaturated fats contain one or more double bonds in their structure. The skilled artisan knows that unsaturated fats can be either polyunsaturated (many double bonds) or monounsaturated fats (one or few double bonds). Thus, the skilled artisan would know structures of any sugar, carbohydrate, starch, polypeptide, amino acid or fat.

In addition to the fact that the skilled artisan would know structures of sugars, carbohydrates, starches, polypeptides, amino acids and fats, the specification discloses specific non-limiting examples of nutrients that can induce production of a protein (see, for example, page 19, lines 14-19), including glucose (see, for example, page 13, lines 21-24; and page 17, lines 17-25) and Vitamin D (page 17, lines 26-28). Thus, in view of the guidance in the

specification and knowledge in the art, the skilled artisan would know generic structures as well as specific examples of nutrients that induce production of a protein.

Nevertheless, without acquiescing to the propriety of the rejection and solely in order to further prosecution of the application, claims 32, 33, 39, 44, 50 and 53 have been cancelled herein without prejudice rendering the rejection moot. The pending and new claims recite, *inter alia*, "sugar, polypeptide, amino acid or fat." The rejection will therefore be addressed insofar as it may pertain to the claims upon entry of the Response.

As discussed above and in the record, in view of the specification and knowledge in the art, one skilled in the art would be apprised of a variety of nutrients that induce production of a protein, by virtue of either increased gut endocrine promoter expression or increased gut endocrine promoter secretion. In support of Applicant's position, the following is a non-limiting list of nutrients that can induce production of a protein from gut endocrine cells:

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|---|---|
| Carbohydrates | Cataland S et. al. (1974) J. Clin. Endocrinol. Metab. 39:223-228<br>Pederson RA et. al. (1975) Diabetes 24:1050-1056<br>Falko et. al. JM (1980) Clin. Endocrinol. 13:587-593 |
| Glucose | Morgan LM et. al. (1978) Ann. Clin. Biochem. 15:172-177 |
| Galactose | Morgan LM et. al. (1978) Ann. Clin. Biochem. 15:172-177 |
| Sucrose | Morgan LM et. al. (1979) Annals Clin. Biochem. 16:6-14 |
| Fructose | Flatt (1989) J. Nutr. 119:1300-1303 |
| α-methyl glucoside | Hopfer U (1987) In: Physiology of the gastrointestinal tract. 2nd ed. LR Johnson (ed) Raven Press, New York, Chpt 55.<br>Flatt (1989) J. Nutr. 119:1300-1303 |
| 3-O-methyl glucose | Hopfer U (1987) In: Physiology of the gastrointestinal tract. 2nd ed. LR Johnson (ed) Raven Press, New York, Chpt 55. |
| 2-deoxyglucose | Flatt (1989) J. Nutr. 119:1300-1303 |
| Fat | Kuzio M et. al. (1974) Gastroenterol. 66:357-364<br>Falko JM et. al. (1975) J. Clin. Endocrinol. Metab. 31:260-265 |
| Triglycerides | Pederson RA et. al. (1975) Diabetes 24:1050-1056 |
| Fatty acids | Brown JC (1974) In: Endocrinology 1973. S. Taylor, ed., Heinemann, London, pp276-284<br>Falko JM et. al. (1975) J. Clin. Endocrinol. Metab. 31:260-265 |
| Saturated fat | Lardinois CK (1988) J. Am. College Nutr. 6:507-515 |
| Monosaturated fat | Lardinois CK (1988) J. Am. College Nutr. 6:507-515 |
| Polysaturated fat | Lardinois CK (1988) J. Am. College Nutr. 6:507-515 |
| Amino acids | Thomas FB et. al. (1976) Gastroenterol. 70:523-527<br>Schulz TB et. al. (1982) Gastroenterol. 17:357-362 |
| Peptone | Wolfe MM and McGuigan JE (1982) Gastroenterol 83:864-872 |

In view of the foregoing, the skilled artisan would know a variety of sugars, polypeptides, amino acids and fats that can induce production of a protein. Consequently, the skilled artisan

would know of various species within the genus of sugars, polypeptides, amino acids and fats that can induce production of a protein.

The Patent Office appears to have taken the position that merely because the genus of nutrients, namely sugars, polypeptides, amino acids and fats, appears broad that the skilled artisan would not know of a number of sugars, polypeptides, amino acids and fats that can induce production of a protein. However, as set forth herein and in the record, in view of the guidance in the specification and knowledge in the art the skilled artisan would know of various sugars, polypeptides, amino acids and fats that can induce production of a protein.

Furthermore, the law does not require disclosure of a minimum number of specific examples of nutrients in order to satisfy the written description requirement under §112, first paragraph. Again, the law only requires that the skilled artisan be apprised, with reasonable clarity, of sugars, polypeptides, amino acids and fats that can induce production of a protein. Here, the guidance in the specification and knowledge in the art are adequate to apprise the skilled artisan of various sugars, polypeptides, amino acids and fats that induce production of a protein. Consequently, because the skilled artisan would know of a variety of sugars, polypeptides, amino acids and fats that induce production of a protein, an adequate written description is provided for claims 31, 34 to 36, 38, 40, 43, 47 to 49, 51, 52, 54, 55, 71 to 73, 76, 78 to 80, 82, 83 and 85 to 113. Accordingly, the rejection under 35 U.S.C. §112, first paragraph, as allegedly lacking an adequate written description is improper and must be withdrawn.

The rejection of claims 31 to 36, 38 to 40, 43, 44 and 47 to 55 under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement is respectfully traversed. The Examiner has maintained the rejection for grounds of record as well as new grounds.

Claims 31 to 36, 38 to 40, 43, 44 and 47 to 55 are adequately enabled prior to entry of the Response. Applicants have provided substantial evidence corroborating that cells can be transformed with a polynucleotide comprising an expression control element in operable linkage with a nucleic acid encoding a protein *in vitro* and *in vivo*, for example, by transplantation of transformed cells, by injection, or by luminal (intra-cavity) incubation, and that transformed cells can produce functional and therapeutically relevant levels of proteins *in vivo* for long periods of time, all in accordance with the invention. Applicants again respectfully remind the Patent Office that the failure to disclose other methods by which the claimed invention may be made

does not render a claim invalid under 35 U.S.C. §112. *Spectra-Physics v. Coherent, Inc.*, 827 F.2d 1524, 1533 (Fed. Cir., *cert. denied*, 484 U.S. 954 (1987)- citing *In re Glass*, 492 F.2d 1228, 1233, "[n]onenablement is the failure to disclose *any* mode and does not depend on the applicant advocating a particular embodiment or method for making the invention.")

Thus, in view of the guidance in the specification, as corroborated by the evidence of record, the skilled artisan could practice claims 31 to 36, 38 to 40, 43, 44 and 47 to 55 without undue experimentation. As such, the claims are adequately enabled and the rejection under 35 U.S.C. §112, first paragraph, is improper and must be withdrawn.

Nevertheless, without acquiescing to the propriety of the rejection and solely in order to further prosecution of the application, claims 32, 33, 39, 44, 50 and 53 have been cancelled herein without prejudice rendering the rejection moot. Claims 31, 34 to 36, 38, 40, 43, 47 to 49, 51, 52, 54 and 55 have been amended to recite, *inter alia*, "a glucose-dependent insulinotropic polypeptide (GIP) promoter or chromogranin A promoter." The claims have also been amended to recite, as appropriate, treating a subject having or at risk of having "diabetes" and "undesirable body mass," in order to "decrease blood glucose" and "treat "undesirable body mass," respectively. The claims have also been amended to recite "wherein the endocrine cell transformation occurs *in vivo* via intra-cavity delivery." The rejection will therefore be addressed insofar as it may pertain to the claims upon entry of the Response.

A brief review of the evidence of record demonstrating that the claims are adequately enabled is as follows:

1) The specification, Example III. This example included studies indicating that human insulin can be produced by gut mucosum of transgenic mice following transformation of a human insulin gene expression construct into fertilized embryos. Amounts of human insulin produced from the gut mucosum of the animals was effective to protect the animals from diabetes following destruction of the endogenous insulin producing pancreatic beta-cells.

2) Previously submitted Exhibit A, a sworn Declaration under 37 C.F.R. §1.132 executed by Dr. Timothy Kieffer and accompanying Figures 1-11, filed June 18, 2003. Exhibit A included animal studies in which leptin expressed by gut cells transformed *in vitro*, when implanted into animals, expressed leptin in an amount effective to cause weight loss and normalize glucose levels in the animals. The data in previously submitted Exhibit A and accompanying Figures 1-11 therefore corroborates that a polynucleotide encoding a protein (e.g.,

leptin) can be introduced into mucosal tissue cells which, when implanted into animals, produce the protein (e.g., leptin) in amounts effective to treat the appropriate disorder (e.g., obesity).

3) Previously submitted Exhibits 1 and 2, a sworn Declaration under 37 C.F.R. §1.132 executed by Dr. Anthony Cheung and accompanying Figures 1-4, respectively, filed June 16, 2004. Exhibits 1 and 2 included data demonstrating *in vivo* transformation of three different genes using two different vectors into gut or gastrointestinal mucosal tissue of animals, via injection or luminal incubation. In brief, *in vivo* mucosal cells transformed with the human insulin gene produced human insulin in animals at levels sufficient to reduce glucose. In these animals, human insulin was produced long term, 128 days following *in vivo* mucosal cell transformation with the human insulin gene. In addition to demonstrating *in vivo* transformation and production of insulin at therapeutically relevant levels, two other genes, red fluorescent protein (DsRed) and green fluorescent protein (GFP), were present in mucosal tissue of animals following *in vivo* mucosal cell transformation using two different vectors (AAV and FIV). The data in previously submitted Exhibits 1 and 2 therefore corroborates that gene transfer into gut or gastrointestinal mucosal tissue *in vivo* is A) independent of the particular gene transferred; and B) can be performed with different vectors.

4) Previously submitted Exhibit AA, a sworn Declaration under 37 C.F.R. §1.132 by Dr. Anthony Cheung and accompanying Figures 1 and 2, filed February 10, 2005. Exhibit AA and Figures 1 and 2 included data demonstrating *in vivo* transformation with two different genes (insulin and SEAP). In brief, *in vivo* mucosal cells were transformed by a single injection of a polynucleotide encoding insulin or SEAP gene, each of which was expressed by a GIP or chromagranin A promoter, into gut lumen. The transformed cells in the animals 1) produced insulin and SEAP long term; 2) produced insulin and SEAP at therapeutically relevant levels, even 150 days after vector delivery; and 3) continued producing insulin 150 days after delivery so that the animals survived STZ- treatment, whereas control animals died within 3 days of STZ treatment, demonstrating that insulin was functional and therapeutic. The data in previously submitted Exhibit A and accompanying Figures 1 and 2, therefore corroborate that genes transferred into gut or gastrointestinal mucosal tissue of animals *in vivo* A) express encoded proteins that are functional and at therapeutic levels; B) express encoded proteins for a long period of time; C) is achievable by a single administration; and D) are expressed using different gut endocrine promoters.

In view of the foregoing evidence in the specification and in the record, that therapeutic levels of insulin and leptin can be expressed in animal gut or gastrointestinal mucosal tissue cells transformed *in vivo* or *in vitro* with a nucleic acid encoding insulin or leptin, using different vectors delivered *in vivo*, and using different gut endocrine promoters, claims 31 to 36, 38 to 40, 43, 44 and 47 to 55, prior to entry of the Response are adequately enabled. Accordingly, the rejection under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement, is improper and must be withdrawn.

Nevertheless, solely in order to further prosecution of the subject application and without acquiescing to the propriety of the rejection, claims 32, 33, 39, 44, 50 and 53 have been cancelled herein without prejudice. The pending and new claims recite, *inter alia* " a glucose-dependent insulinotropic polypeptide (GIP) promoter or chromagranin A promoter," and that the subject has or is at risk of having "diabetes" or "undesirable body mass." The pending and new claims recite that the methods either "decrease blood glucose" or treat "undesirable body mass." The pending and new claims recite that "the endocrine cell transformation occurs *in vivo* via intra-cavity delivery." New claims 89 to 102 are directed to subject matter covered by the elected claims prior to entry of the amendments set forth above, namely "transforming a gut or gastrointestinal mucosal tissue endocrine cell *in vitro* with a polynucleotide comprising a glucose-dependent insulinotropic polypeptide (GIP) promoter or chromagranin A promoter in operable linkage with a nucleic acid encoding insulin," followed by "implanting the cell transformant" that "produces insulin" into "gut or gastrointestinal mucosal tissue of a subject." New claims 103 to 113 are directed to subject matter covered by the elected claims prior to entry of the amendments set forth above, namely "transforming a gut or gastrointestinal mucosal tissue endocrine cell *in vitro* with a polynucleotide comprising a glucose-dependent insulinotropic polypeptide (GIP) promoter or chromagranin A promoter in operable linkage with a nucleic acid encoding leptin," followed by "implanting the cell transformant" that "produces leptin" into "gut or gastrointestinal mucosal tissue of a subject."

In view of the claim amendments and language of the new claims, claims 31, 34 to 36, 38, 40, 43, 47 to 49, 51, 52, 54, 55, 71 to 73, 76, 78 to 80, 82, 83 and 85 to 113 are adequately enabled in view of the guidance in the specification, as corroborated by the evidence of record discussed in brief above. Applicants will therefore only address the new grounds for the rejection set forth in the Office Action at pages 9-11.

With respect to the STZ animal model, Applicants recognize that human diabetes is not typically caused by ingesting STZ. However, STZ need not be a real world cause of hyperglycemia and diabetes in order for the STZ animal model to be a model reasonably predictive of treatments for hyperglycemia and diabetes. The STZ animal model is reasonably predictive of treatments because it reflects a reduction or loss of insulin in animals including humans that leads to hyperglycemia and development of diabetes, which can be due to loss or destruction of insulin producing pancreatic beta-cells. The loss of insulin and consequent development of hyperglycemia and diabetes in STZ treated animals is analogous to loss of insulin and development of hyperglycemia and diabetes in humans. In other words, in both humans and STZ treated animals a loss of insulin produces the same result: hyperglycemia and development of diabetes. Thus, treatments effective for STZ animals are reasonably predictive of effective human treatments. In support of Applicants' position, insulin treatment of human diabetics and STZ animals decreases blood glucose levels in human diabetics and in STZ animals. Thus, the fact that insulin is effective as a treatment in both human diabetes and STZ induced diabetes further demonstrates that the STZ animal model is reasonably predictive of hyperglycemia and diabetes treatments in animals, including humans.

With respect to leptin, and that increased leptin allegedly would not be predicted to treat obese children that already express increased levels of leptin, Applicants respectfully point out that subjects that express leptin can be treated by providing leptin. Thus, even if a subject produces leptin, such subjects can be treated in accordance with the invention methods.

To corroborate Applicants' position that a subject that expresses leptin can be treated in accordance with the invention methods, submitted herewith as Exhibit A, a publication by Heymsfield et al. (JAMA 282:1568 (1999)). In Exhibit A, the authors report the effect of subcutaneous leptin administration on both lean and obese subjects. The authors report that weight loss occurred in both lean and obese subjects (see results, page 1568). The authors also report that leptin appeared to induce weight loss in obese subjects with elevated endogenous serum leptin levels (see Conclusions, page 1568), weight loss was observed in obese subjects that expressed endogenous leptin. Consequently, in view of Exhibit A, a subject that expresses leptin, including an obese subject, can be treated in accordance with the invention methods.

In view of the foregoing, claims 31, 34 to 36, 38, 40, 43, 47 to 49, 51, 52, 54, 55, 71 to 73, 76, 78 to 80, 82, 83 and 85 to 113 are adequately enabled. Accordingly, the rejection under

35 U.S.C. §112, first paragraph, as allegedly lacking enablement, is improper and must be withdrawn.

II. REJECTIONS UNDER 35 U.S.C. §102(b) and §103(a)

The rejection of claims 31 to 34, 36, 38, 40, 43, 47 to 50, 54 and 55 under 35 U.S.C. §102(b) as allegedly anticipated by German *et al.* (U.S. Patent No. 5,837,693) is respectfully traversed. Allegedly, German *et al.* describe "genetically-altered secretory gland cells which are genetically altered to incorporate a gene which expresses a protein, for the treatment of diseases....one disease which may be treated is diabetes, by the expression of insulin." Allegedly, German *et al.* describe "mucosal secretion....mucosal cells of the gastrointestinal tract," and "promoters inducible by external agents....and gut endocrine promoters." Allegedly, "such cells may be endocrine cells, as such would be required for secretion." [Office Action, pages 11-12]

Anticipation requires the disclosure in a single prior art reference of each element of the claim under consideration *In re Spada*, 911 F.2d 705 (Fed. Cir. 1990), *In re Bond*, 910 F.2d 831 (Fed. Cir. 1990).

As a first issue, Applicants respectfully point out that a reference cited under 35 U.S.C. §102(b) must have an enabling disclosure. *citations omitted*, see, M.P.E.P. §2121. Thus, for the rejection to be proper, German *et al.* must enable rejected claims 31 to 34, 36, 38, 40, 43, 47 to 50, 54 and 55. However, these claims have been rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement. Consequently, the rejections under 35 U.S.C. §102(b) and 35 U.S.C. §112, first paragraph, are contradictory and cannot be maintained simultaneously. If the claims are enabled by German *et al.* as required for a rejection to be proper under 35 U.S.C. §102(b), then the rejection under 35 U.S.C. §112, first paragraph must be withdrawn. Applicants therefore respectfully request that the Patent Office withdraw either the rejection under 35 U.S.C. §102(b) or the rejection under 35 U.S.C. §112, first paragraph.

As a second issue, Applicants respectfully disagree with the alleged teachings of German *et al.* set forth in the Office Action. For example, gut endocrine promoters and gut or gastrointestinal endocrine cells are neither taught nor suggested by German *et al.* In this regard, German *et al.* describe salivary and pancreatic amylase promoters and insulin promoter (see, for example, column 4, lines 28-33). Salivary and pancreatic amylase promoters are exocrine gland

promoters, not gut endocrine promoters. Insulin promoter is active in pancreatic beta cells, not in gut or gastrointestinal endocrine cells. German *et al.* describe other non-gut endocrine promoters including CMV, MMTV, RSV and AV virus promoters (column 7, lines 14-28; Figure 1). Furthermore, German *et al.* in all actual or hypothetical studies, employ a vector in which gene expression is driven by non-gut endocrine promoters, namely CMV promoter (pBAT14.hIns; (Examples 1, 2 and 5), a salivary amylase promoter (Example 6), a moloney-LTR promoter (Example 7), an RSV LTR promoter (Example 8), a pancreatic α amylase promoter (Examples 9 and 11), or a human insulin promoter (Example 10). As additional evidence that German *et al.* fail to teach or suggest gut endocrine promoters or gut or gastrointestinal endocrine cells, the cholinergic agonist acetyl-β-methyl choline (McH) used by German *et al.* stimulates exocrine cell secretion, not endocrine cell secretion (see, Examples 1 to 4). Thus, in view of the foregoing deficiencies, German *et al.* fail to teach or suggest gut endocrine promoters or gut or gastrointestinal endocrine cells.

As further evidence that German *et al.* fail to teach or suggest gut endocrine promoters or gut or gastrointestinal endocrine cells, submitted herewith is Exhibit B, a publication by Goldfine *et al.*, in which German is listed as a co-author (Nature Biotechnol. 15:1378 (1997)). In the Abstract and Introduction sections of Exhibit B, the authors state that the "exocrine pancreas, liver and submandibular glands of the rat were used to express and secrete....human growth hormone and insulin." [*Emphasis added*] The authors also state that "these three glands can be accessed noninvasively by....administration of DNA vectors directly into their duct systems" (page 1378, column 1, second paragraph, *Emphasis added*). Exocrine glands have ducts indicating that the transformed cells are exocrine cells, not endocrine cells. Further in this regard, the authors of Exhibit B report that "transfection of the exocrine glands of the gastrointestinal tract can be effective...." (page 1378, column 2, first paragraph) Exhibit B therefore corroborates that German *et al.* transforms exocrine cells, not endocrine cells. Consequently, as corroborated by Exhibit B German *et al.* fail to teach or suggest gut or gastrointestinal endocrine cells, let alone gut endocrine promoters.

As a third issue, German *et al.* is not an enabling disclosure as is required for a reference to be properly cited under 35 U.S.C. §102(b). In this regard, the data in German *et al.* do not demonstrate that *in vivo* gene transfer, protein expression and secretion from exocrine cells *in*

*vivo* was successful, let alone in amounts effective to treat a subject. For example, in German *et al.* at Table 3, column 20, there was no meaningful difference in insulin levels between transformed and control animals induced with McH (compare cDNA1 and cDNA2 vs. control1 and control2; cDNA1, 10.0 and cDNA2 11.6, vs. control 2, 9.2). Furthermore, at Tables 4 and 5, column 21, no control was used in these studies making it unclear whether insulin levels increased, particularly in view of Table 5, which shows that insulin levels for cDNA5 actually decreased after IsO injection: 8.5 vs. 8.0. Furthermore, at Table 6, column 22, glucose levels increased after McH induction (385 vs. 268 and 413 vs. 321), which is the opposite of what one would expect if insulin was secreted by transformed exocrine cells, i.e., glucose levels should have decreased. In view of the poor data, it is highly unlikely that German *et al.* achieved *in vivo* gene transfer, protein expression and secretion from exocrine cells, let alone at levels effective to treat a subject, particularly in view of the fact that glucose levels actually increased in animals (Table 6, column 22). German *et al.* therefore fails to be an enabling disclosure as is required of a reference cited under 35 U.S.C. §102(b).

Nevertheless, without acquiescing to the propriety of the rejection and solely in order to further prosecution of the application, claims 32, 33 and 50 have been cancelled herein without prejudice. The pending and new claims recite, *inter alia*, "a glucose-dependent insulinotropic polypeptide (GIP) promoter or chromogranin A promoter." As discussed above, German *et al.* fail to teach or suggest a gut endocrine promoter, let alone a glucose-dependent insulinotropic polypeptide (GIP) promoter or a chromogranin A promoter and, therefore, cannot anticipate the claims. Accordingly, the rejection under 35 U.S.C. §102(b) is improper and must be withdrawn.

The rejection of claims 31, 43, 44 and 51 under 35 U.S.C. §103(a) as allegedly unpatentable over German *et al.* (U.S. Patent No. 5,837,693) and Boylan *et al.* (J. Biol. Chem. 272:17438 (1997)) is respectfully traversed. Allegedly, German *et al.* describe "genetically-altered secretory gland cells which are genetically altered to incorporate a gene which expresses a protein, for the treatment of diseases....one disease which may be treated is diabetes, by the expression of insulin." Allegedly, German *et al.* describe "mucosal secretion....mucosal cells of the gastrointestinal tract," and "promoters inducible by external agents....and gut endocrine promoters." German *et al.* is acknowledged not to teach the use of GIP promoter. Allegedly,

Boylan *et al.* describe the "structure of GIP promoter and....that such promoter is responsive to glucose levels....in K cells of the duodenum and jejunum." [Office Action, pages 13-14]

Claims 31, 43, 44 and 51 would not have been obvious under 35 U.S.C. §103(a) over German *et al.* (U.S. Patent No. 5,837,693) and Boylan *et al.* (J. Biol. Chem. 272:17438 (1997)). In order for a rejection to be proper under 35 U.S.C. §103(a), *inter alia*, there must have been 1) a motivation to combine the references at the time of the invention; 2) the combination of references must teach or suggest each and every element of the claimed invention; and 3) there must have been a reasonable expectation of success at the time of the invention. Both the teaching or suggestion to make the claimed combination and the reasonable expectation of success must be found in the prior art, not in Applicants' disclosure. See, e.g., *In re Vaeck*, 947 F.2d 488 (Fed. Cir. 1991); and *In re O'Farrell*, 853 F.2d 894, 903-904 (Fed. Cir. 1988).

As a first issue, Applicants respectfully point out that the rejections under 35 U.S.C. §103(a) and 35 U.S.C. §112, first paragraph are contradictory. Under 35 U.S.C. §103(a), *inter alia*, the cited reference(s) must provide a reasonable expectation of success of producing the claimed methods. Here, claims 31, 43, 44 and 51 have been rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement. Consequently, if there was a reasonable expectation of success of producing claims 31, 43, 44 and 51 as required for a rejection to be proper under 35 U.S.C. §103(a), then claims 31, 43, 44 and 51 should be enabled under 35 U.S.C. §112, first paragraph. Applicants therefore respectfully request that the Patent Office clarify the contradiction or withdraw either the rejection under 35 U.S.C. §103(a) or the rejection under 35 U.S.C. §112, first paragraph.

As discussed above, German *et al.* transform exocrine cells, not endocrine cells (see, also, Exhibit B). German *et al.* also fail to teach or suggest gut endocrine promoters. Boylan *et al.* report that GIP promoter is active in a neuroendocrine cell line. However, Boylan *et al.* report that GIP promoter is inactive in exocrine cells derived from salivary gland, namely SCA-9 and Hs124 (see page 17440, first column, under RESULTS). In particular, the authors state that "[e]xpression of GIP was demonstrated only in the mouse neuroendocrine tumor cell line STC-1 and the human embryonic intestinal 407 cell line." [Emphasis added] Thus, because German *et al.* transform exocrine cells, and fail to teach or suggest an endocrine cell promoter, let alone a GIP promoter, and Boylan *et al.* report that GIP promoter is inactive in two exocrine cell types, there is no reason why the skilled artisan would use Boylan *et al.*'s GIP promoter with German *et*

*al.* to express protein in exocrine cells. In this regard, the skilled artisan would not have been motivated at the time of the invention to use GIP promoter to express a protein in exocrine cells because Boylan *et al.* report that GIP promoter was not active in two types of exocrine cells. Consequently, there would not have been any motivation to combine Boylan *et al.* with German *et al.* at the time of the invention.

Moreover, because Boylan *et al.* report that GIP promoter was not active in exocrine cells derived from salivary gland, the skilled artisan would not have had a reasonable expectation of success that an endocrine cell promoter could confer protein expression in exocrine cells at all, let alone in an amount effective to treat a subject. Consequently, even if Boylan *et al.* were combined with German *et al.* the skilled artisan would not have had a reasonable expectation of success at the time of the invention that a protein could be expressed in exocrine cells using GIP promoter at all, let alone at amounts effective to treat a subject, because Boylan *et al.* report that GIP promoter was not active in exocrine cells.

The absence of motivation to use GIP promoter for protein expression in exocrine cells and reasonable expectation of success that the GIP promoter would confer protein expression in exocrine cells at all, let alone *in vivo* in an amount effective to treat a subject, is corroborated by German *et al.*'s own data in which levels of insulin in animals produced by transformed exocrine cells using a CMV promoter did not decrease glucose *in vivo* (as discussed above, glucose levels actually increased in the animals). Consequently, German *et al.* alone and in combination with Boylan *et al.* fail to provide the requisite motivation and requisite reasonable expectation of success at the time of the invention for a proper rejection under 35 U.S.C. §103(a).

Nevertheless, without acquiescing to the propriety of the rejection and solely in order to further prosecution of the application, the pending and new claims recite, *inter alia*, "endocrine cells." As discussed above, German *et al.* fail to teach or suggest transforming endocrine cells to produce insulin, let alone provide a reasonable expectation that insulin produced by endocrine cells *in vivo* would be effective to decrease blood glucose in animals. Furthermore, German *et al.* in combination with Boylan *et al.* fail to provide the requisite motivation and reasonable expectation of success that insulin can be produced by transformed cells at all, let alone *in vivo* in an amount effective to decrease blood glucose in animals. Accordingly, the rejection under 35 U.S.C. §103(a) is improper and must be withdrawn.

## CONCLUSION

In summary, for the reasons set forth herein, Applicants maintain that claims 31, 34 to 36, 38, 40, 43, 47 to 49, 51, 52, 54, 55, 71 to 73, 76, 78 to 80, 82, 83 and 85 to 113 clearly and patentably define the invention, respectfully request that the Examiner reconsider the various grounds set forth in the Office Action, and respectfully request the allowance of the claims which are now pending.

If the Examiner would like to discuss any of the issues raised in the Office Action, Applicant's representative can be reached at (858) 509-4065.

Please charge any fees associated with the submission of this paper to Deposit Account Number 502212. The Commissioner for Patents is also authorized to credit any over payments to the above-referenced Deposit Account.

Respectfully submitted,

PILLSBURY WINTHROP SHAW PITTMAN LLP

ROBERT M. BEDGOOD
Reg. No. 43488
Tel. No. 858 509.4065
Fax No. 858 509.4010

Date: October 6, 2005
11682 El Camino Real
Suite 200
San Diego, CA 92130-2092
(619) 234-5000


**CERTIFICATION UNDER 37 C.F.R. §§ 1.8 and/or 1.10***

(When using Express Mail, the Express Mail label number is ***mandatory;Express Mail certification is optional.***)

I hereby certify that, on the date shown below, this paper (along with any paper referred to as being attached or enclosed) is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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Date: October 6, 2005

PATRICIA MUNOZ
*(type or print name of person certifying)*

** Only the date of filing (§ 1.6) will be the date used in a patent term adjustment calculation, although the date on any certificate of mailing or transmission under § 1.8 continues to be taken into account in determining timeliness. See § 1.703(f). Consider "Express Mail Post Office to Addressee" (§ 1.10) or facsimile transmission (§ 1.6(d)) for the reply to be accorded the earliest possible filing date for patent term adjustment calculations.*

PRELIMINARY COMMUNICATION

# Recombinant Leptin for Weight Loss in Obese and Lean Adults

## A Randomized, Controlled, Dose-Escalation Trial

Steven B. Heymsfield, MD
Andrew S. Greenberg, MD
Ken Fujioka, MD
Russell M. Dixon, MD
Robert Kushner, MD
Thomas Hunt, MD
John A. Lubina, PhD
Janet Patane, MPH
Barbara Self, MPH
Pam Hunt, PhD
Mark McCamish, PhD, MD

**Context** The protein hormone leptin is important to the homeostatic regulation of body weight. Treatment with exogenous leptin may affect weight loss.

**Objective** To determine the relationship between increasing doses of exogenous leptin administration and weight loss in both lean and obese adults.

**Design** A randomized, double-blind, placebo-controlled, multicenter, escalating dose cohort trial conducted from April 1997 to October 1998.

**Setting** Four university nutrition and obesity clinics and 2 contract clinical research clinics.

**Participants** Fifty-four lean (body mass index, 20.0-27.5 kg/m$^2$; mean [SD] body weight, 72.0 [9.7] kg) and 73 obese (body mass index, 27.6-36.0 kg/m$^2$; mean [SD] body weight, 89.8 [11.4] kg) predominantly white (80%) men (n = 67) and women (n = 60) with mean (SD) age of 39 (10.3) years.

**Interventions** Recombinant methionyl human leptin self-administered by daily morning subcutaneous injection (0 [placebo], 0.01, 0.03, 0.10, or 0.30 mg/kg). In part A, lean and obese subjects were treated for 4 weeks; in part B, obese subjects were treated for an additional 20 weeks. Lean subjects consumed a eucaloric diet to maintain body weight at the current value, and obese subjects were prescribed a diet that reduced their daily energy intake by 2100 kJ/d (500-kcal/d) from the amount needed to maintain a stable weight.

**Main Outcome Measures** Body weight, body fat, and incidence of adverse events.

**Results** Weight loss from baseline increased with increasing dose of leptin among all subjects at 4 weeks ($P$ = .02) and among obese subjects at 24 weeks ($P$ = .01) of treatment. Mean (SD) weight changes at 4 weeks ranged from −0.4 (2.0) kg for placebo (n = 36) to −1.9 kg (1.6) kg for the 0.1 mg/kg dose (n = 29). Mean (SD) weight changes at 24 weeks ranged from −0.7 (5.4) kg for the 0.01 mg/kg dose (n = 6) to −7.1 (8.5) kg for the 0.30 mg/kg dose (n = 8). Fat mass declined from baseline as dose increased among all subjects at 4 weeks ($P$ = .002) and among obese subjects at 24 weeks of treatment ($P$ = .004); more than 95% of weight loss was fat loss in the 2 highest dose cohorts at 24 weeks. Baseline serum leptin concentrations were not related to weight loss at week 4 ($P$ = .88) or at week 24 ($P$ = .76). No clinically significant adverse effects were observed on major organ systems. Mild-to-moderate reactions at the injection site were the most commonly reported adverse effects.

**Conclusions** A dose-response relationship with weight and fat loss was observed with subcutaneous recombinant leptin injections in both lean and obese subjects. Based on this study, administration of exogenous leptin appears to induce weight loss in some obese subjects with elevated endogenous serum leptin concentrations. Additional research into the potential role for leptin and related hormones in the treatment of human obesity is warranted.

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THE PROTEIN HORMONE LEPTIN, encoded by the *obese* gene and produced by adipose tissue,[1-6] appears to signal adiposity and modulate ingestive behavior. Several lines of evidence support this conclusion: exogenous leptin administration results in a loss of body fat in ani-

**Author Affiliations:** Weight Control Unit, St Luke's-Roosevelt Hospital, New York, NY (Dr Heymsfield); Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging, Tufts University, New England Medical Center, Boston, Mass (Dr Greenberg); Scripps Clinic, San Diego, Calif (Dr Fujioka); Covance Inc and University of Wisconsin, Madison (Dr Dixon); Northwestern University, Chicago, Ill (Dr Kushner); and PPD Pharmaco Inc, Austin, Tex (Dr T. Hunt); and Departments of Biostatistics (Dr Lubina), Clinical Affairs (Ms Patane), International Clinical Safety (Ms Self), Product Development (Dr P. Hunt), and Clinical Research (Dr McCamish), Amgen Inc, Thousand Oaks, Calif.

**Financial Disclosures:** Drs Lubina, Patane, P. Hunt, and McCamish and Ms Self are employees of Amgen Inc, which manufactures the recombinant methionyl human leptin used in this study.

**Corresponding Author and Reprints:** Steven B. Heymsfield, MD, Weight Control Unit, St Luke's-Roosevelt Hospital, 1090 Amsterdam Ave, New York, NY 10025 (e-mail: SBH2@Columbia.edu).

mals[2,3,7,8]; animals and humans who have a genetic deficiency of leptin exhibit extreme obesity[1,5]; and serum concentrations of leptin increase with body fat in very obese persons who do not have a genetic mutation.[6,9-12] Leptin levels and body fat are highly correlated, and body fat accounts for approximately 50% to 60% of the variation in serum leptin concentrations among people.[13] Other factors (eg, sex, diurnal variation, and serum insulin concentration) correlate to a lesser extent.[13,14] Leptin concentrations in the cerebrospinal fluid (CSF) increase with body fat[15-17] but are generally 2 orders of magnitude lower than serum concentrations. The ratio of CSF to serum leptin concentrations also appears to be lower in obese subjects.[15,16] These relationships suggest that administering exogenous leptin might affect homeostatic mechanisms of energy regulation to alter body weight. Alternatively, the higher serum leptin concentrations in obese subjects may suggest that exogenous leptin administration would be ineffective in decreasing adiposity.[15]

We report herein the effects of an exogenously administered recombinant leptin, studied in a randomized, double-blind, placebo-controlled, escalating-dose cohort trial in lean and obese adult subjects. The hypothesis was tested that increasing doses of exogenous leptin administration would result in dose-dependent weight loss in both lean and obese adults.

## METHODS

### Study Design

The study was conducted from April 1997 to October 1998 at 4 university nutrition and obesity clinics and 2 contract clinical research clinics. Subjects enrolled at the university clinics were selected from the investigators' patient populations; subjects at the contract clinical research clinics were selected from their client databases. The hypothesis was tested by monitoring body weight and composition changes among subjects randomly assigned to escalating dose groups of recombinant methionyl human leptin (rL) (Amgen Inc, Thousand Oaks, Calif) or matching placebo (sorbitol and sodium acetate, pH 4.0). Subcutaneous bolus injections (0.01, 0.03, 0.10, or 0.30 mg/kg per day or placebo) were prescribed to 2 strata of lean subjects whose body mass index (BMI), calculated as weight in kilograms divided by the square of height in meters, was 20.0 to 23.4 kg/m$^2$ and 23.5 to 27.5 kg/m$^2$ and to 2 strata of overweight and obese subjects whose BMI was 27.6 to 30.0 kg/m$^2$ and 30.1 to 36.0 kg/m$^2$, respectively. For simplicity, the latter 2 cohorts are collectively referred to as *obese*. Subjects were healthy lean and obese adults. Those with comorbidities of obesity, especially drug-treated diabetes, hyperlipidemia, and hypertension, were excluded. Women were postmenopausal or surgically sterile. All subjects provided written informed consent. An investigational review board at each study site approved the protocol.

As part of the dose escalation, some subjects received rL by subcutaneous continuous infusion at dosages of up to 2 mg/kg per day, which produced entirely different pharmacokinetics. This report summarizes results of subjects given bolus injections. One group of subjects treated with 0.3 mg/kg per day of rL provided at a higher concentration experienced unacceptable reactions at the injection site (eg, ecchymosis, erythema, pruritus), referred to collectively as *injection site reactions* (ISRs). Enrollment in this group was halted by the data monitoring committee after 11 subjects were treated for 4 weeks or less. The study drug was discontinued, and the results from these subjects are not included.

This study was conducted in 2 parts. Dose response to rL was evaluated in both lean and obese subjects for 4 weeks (part A). Because the effects of rL were unknown and prolonged weight loss was not desirable in lean subjects, only obese subjects were allowed to continue for an additional 20 weeks (part B). Lean subjects in part A were maintained on a eucaloric diet, a diet that maintains body weight at the current value, and obese subjects in parts A and B were prescribed a diet that reduced their daily energy intake by 2100 kJ/d (500 kcal/d) from the amount needed to maintain a stable weight with nutritional counseling; 3-day food intake diaries were used to monitor subject adherence to the diet. Obese subjects were encouraged to walk briskly for 20 to 30 minutes, 3 to 5 times per week. Blinded study drug (placebo and rL) was self-administered subcutaneously once daily before 11 AM. Compliance was assessed by accounting for vials of study drug used.

### End Points and Measures

Body weight, measured to the nearest 0.1 kg on calibrated scales, was the primary end point. Body composition (weight, fat mass, and fat-free mass) was determined by dual x-ray absorptiometry (DXA) using Lunar DPX densitometers (Lunar Corporation, Madison, Wis).[18,19] All sites used the same model densitometers, software, and scan mode. DXA systems were calibrated, and scans were analyzed by a central reading laboratory (Bone Fide Ltd, Madison, Wis). To control for hydration status, subjects were instructed not to eat or drink anything other than small amounts of water for at least 8 hours prior to the scan and to avoid strenuous exercise or ingestion of alcohol in the 24 hours prior to the scan.

A central service (Professional Nutrition Systems, Westwood, Kan) estimated energy intake from subjects' food diaries of the 48 hours before a clinic visit. Fasting glucose and insulin concentrations were measured periodically throughout the study. An oral glucose tolerance test (OGTT) (75 g of glucose) was performed at baseline and at the end of parts A (4 weeks) and B (24 weeks). Clinical safety and tolerability evaluations done throughout the study included performing physical examinations and electrocardiograms; checking for adverse events; and measuring serum chemistries, complete blood cell counts, hormone levels (luteinizing hormone, follicle-stimulating hormone, cortisol, and prolactin), and vital signs. In this multicenter trial, clinical chemistry analyses were conducted at a College of American Pathologists–certified

central laboratory (MRL Medical Research Laboratory, Highland Heights, Ky). Serum leptin concentrations were determined by an enzyme-linked immunosorbent assay with a detection limit of 0.04 ng/mL; the assay does not distinguish between endogenous leptin and rL. The presence of serum antibodies against rL was assessed using a solid-phase radioimmunoassay using protein A tagged with iodine 125 to detect IgG bound to rL. Injection site reactions were graded as mild (easily tolerated), moderate (some discomfort), or severe (severe discomfort).

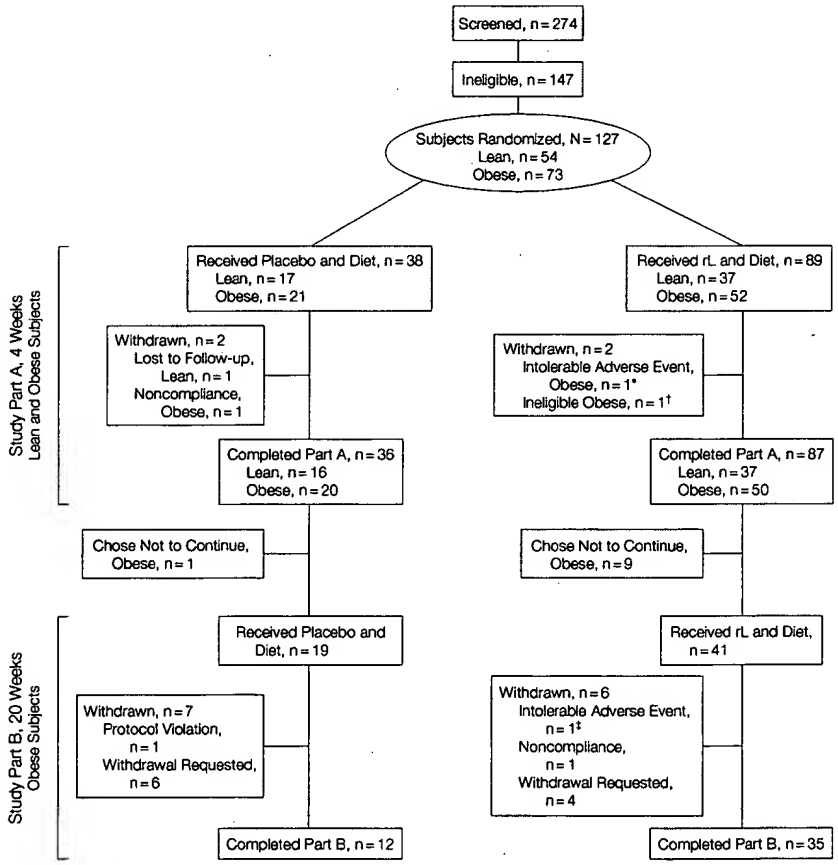
### Statistical Methods

This study was designed to assess safety and to assess dose-response relationships. At each dose, subjects across all BMI groups were pooled to assess safety. After 8 subjects who were taking the study drug had completed 2 weeks of treatment, the study's safety monitoring committee—1 scientist, 2 physicians, and 2 statisticians—reviewed the clinical data. In the absence of any unexpected or clinically significant findings, subject enrollment in the next dose cohort was permitted. After the safety assessment, a 6-subject cohort (4 active and 2 placebo) within each BMI strata was evaluated for weight loss at 2 weeks. If the weight loss difference between active and placebo was less than 0.5 kg or greater than 1.5 kg, no additional subjects were to be enrolled in that dose group. If weight loss effects were between these limits, an additional 6-subject cohort could be enrolled to further characterize weight loss. The operating characteristics of this design ensured an adverse event incidence of at least 30% would be detected with 95% probability. Simulations indicate that this design has at least 80% power to detect a true difference in weight loss of 1.0 kg between active and placebo groups.

All randomly assigned subjects who received at least 1 dose of the study medication were included in these analyses. The primary analysis included data for all subjects with measurements at each time point. As a means of evaluating the robustness of the analysis, a secondary analysis used the last observation carried forward method to impute data for subjects who withdrew prematurely.[20] Results are expressed as mean (SD), unless otherwise noted.

Dose-response relationships were established by simple linear regression methods. The statistical assumptions for regression analyses were met. Subjects in the placebo group were pooled for analysis and assigned a dose of 0. Inferential analyses were assessed at the $P = .05$ significance level. Unplanned multiple comparisons against the placebo group were adjusted using Dunnett's method. Statistical software SAS version 6.12 and JMP version 3.2.2 (SAS Institute, Cary, NC) were used to perform the analyses.

**Figure 1.** Subject Disposition in 2 Study Phases




The 2-part study included lean and obese subjects. Lean subjects were treated with a weight maintaining diet and obese subjects were treated with a reduced-energy diet. Both groups received either placebo or recombinant methionyl human leptin (rL) for 4 weeks (part A). The second part of the study included only obese subjects who were similarly treated for 20 weeks (part B). Each weight category includes 2 weight ranges (see "Methods" section). The asterisk indicates a subject in the 0.1-mg/kg per day dose group who withdrew because of injection site reactions. The dagger indicates a subject in the 0.1-mg/kg dose group who had heart palpitations that were considered unlikely to be related to rL treatment. The double dagger indicates subject withdrew from the 0.01-mg/kg per day dose group because of injection site reactions.

## RESULTS

### Subject Characteristics and Disposition

Of the 274 subjects assessed for inclusion in the study, 147 were ineligible (FIGURE 1). The subject pool at randomization consisted of 127 subjects. The mean (SD) body weight of the 54 lean subjects was 72.0 (9.7) kg and was 89.8 (11.4) kg for the 73 obese subjects. (TABLE 1). At baseline, subject

characteristics were comparable between lean and obese subjects (except body weight) and among dose cohorts (Table 1). The distribution of body weights were balanced within each dose cohort. Sixty of the 70 obese subjects who completed part A continued into part B (Figure 1 and TABLE 2). Eight of the 10 obese subjects who chose not to continue in part B had been enrolled in the 0.3-mg/kg dose cohort (1 placebo-treated, 7 rL-treated) and received the highest injection volumes. Seven of the 9 obese subjects in the placebo group who dropped out did so in part B.

**Table 1.** Baseline Demographics*

| Characteristic | All Subjects | Placebo | rL Dosage, mg/kg per Day 0.01 | 0.03 | 0.10 | 0.30 |
|---|---|---|---|---|---|---|
| **Lean and Obese Subjects in Part A (4 Weeks)** | | | | | | |
| No. of subjects | 127 | 38 | 16 | 16 | 31 | 26 |
| Sex, No. (%) | | | | | | |
| Female | 60 (47) | 16 (42) | 8 (50) | 9 (56) | 14 (45) | 13 (50) |
| Male | 67 (53) | 22 (58) | 8 (50) | 7 (44) | 17 (55) | 13 (50) |
| Race, No. (%) | | | | | | |
| White | 102 (80) | 28 (74) | 15 (94) | 12 (75) | 25 (81) | 22 (85) |
| Hispanic | 9 (7) | 2 (5) | 1 (6) | 1 (6) | 3 (10) | 2 (8) |
| Black | 15 (12) | 7 (18) | 0 (0) | 3 (19) | 3 (10) | 2 (8) |
| Other | 1 (1) | 1 (3) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Age, y | 39.2 (9.9) | 39.8 (8.5) | 39.8 (8.3) | 41.3 (11.9) | 36.8 (10.2) | 39.3 (11.1) |
| Weight, kg | 82.3 (13.9) | 82.8 (15.8) | 83.6 (16.4) | 79.8 (12.5) | 82.1 (10.8) | 82.3 (14.0) |
| BMI, kg/m$^2$ | 27.9 (4.0) | 27.9 (4.5) | 27.6 (4.5) | 27.5 (3.9) | 27.9 (3.6) | 28.1 (3.7) |
| Body fat, % | 33.9 (10.3) | 33.9 (9.6) | 34.6 (10.4) | 35.3 (11.1) | 32.3 (11.4) | 34.4 (10.1) |
| **Obese Subjects in Part B (20 Weeks)** | | | | | | |
| No. of subjects | 60 | 19 | 8 | 8 | 14 | 11 |
| Sex, No. (%) | | | | | | |
| Female | 34 (57) | 9 (47) | 4 (50) | 5 (63) | 10 (71) | 6 (55) |
| Male | 26 (43) | 10 (53) | 4 (50) | 3 (38) | 4 (29) | 5 (46) |
| Race, No. (%) | | | | | | |
| White | 47 (78) | 13 (68) | 8 (100) | 5 (63) | 11 (79) | 10 (91) |
| Hispanic | 3 (5) | 1 (5) | 0 (0) | 0 (0) | 1 (7) | 1 (9) |
| Black | 10 (17) | 5 (26) | 0 (0) | 3 (38) | 2 (14) | 0 (0) |
| Age, y | 40.9 (8.1) | 41.9 (6.2) | 41.1 (7.7) | 38.1 (10.1) | 40.3 (8.1) | 41.7 (10.6) |
| Weight, kg | 89.9 (11.7) | 93.5 (13.9) | 95.8 (12.4) | 87.5 (7.6) | 85.5 (10.1) | 88.0 (10.0) |
| BMI, kg/m$^2$ | 30.7 (2.2) | 31.2 (2.7) | 31.4 (2.6) | 31.0 (1.9) | 30.0 (1.9) | 30.3 (1.0) |
| Body fat, % | 39.6 (6.6) | 38.2 (6.7) | 41.6 (5.5) | 42.1 (8.3) | 39.0 (6.0) | 39.5 (6.9) |

*rL indicates recombinant methionyl human leptin; BMI, body mass index. Data are presented as mean (SD) unless otherwise indicated.

**Table 2.** Subject Disposition by Dose Cohort*

| Dosage, mg/kg per Day | No. of Subjects: Part A (4 Weeks) Enrolled in | Part A (4 Weeks) Completed | Part B (20 Weeks) Eligible for | Part B (20 Weeks) Continued | Part B (20 Weeks) Completed |
|---|---|---|---|---|---|
| Placebo | 38 | 36 | 20 | 19 | 12 |
| 0.01 | 16 | 16 | 8 | 8 | 6 |
| 0.03 | 16 | 16 | 8 | 8 | 8 |
| 0.10 | 31 | 29 | 16 | 14 | 13 |
| 0.30 | 26 | 26 | 18 | 11 | 8 |
| **Total** | **127** | **123** | **70** | **60** | **47** |

*Both lean and obese subjects participated in part A. Only obese subjects were eligible for part B.

## Weight Loss and Body Composition

At the conclusion of part A (4 weeks' treatment), absolute weight changes across the doses studied averaged between −0.4 and −1.9 kg (mean [SD] weight change: placebo [n = 36], −0.4 [2.0] kg; 0.30-mg/kg rL dose, [n = 26], 1.5 [2.0] [−2.0] kg, FIGURE 2). At the conclusion of part B (24 weeks' treatment), absolute weight changes across the doses studied averaged between −0.7 and −7.1 kg with greatest average weight loss in the highest dose cohort (mean [SD] weight change: placebo [n = 12], −1.3 [4.9] kg; 0.30-mg/kg rL dose, [n = 8], −7.1 [8.5] kg; Figure 2).

There were statistically significant dose responses for weight loss from baseline among those who completed 4 weeks of treatment (53 lean and 70 obese subjects, $P = .02$) and 24 weeks of treatment (47 obese subjects, $P = .01$, FIGURE 3). The relationship between escalating dose and weight loss was corroborated using a last observation carried forward analysis (TABLE 3). There was a statistically significant difference in weight loss across doses between lean and obese subjects at 4 weeks ($P = .03$); lean subjects lost about the same amount of weight at all doses. There was no statistically significant relationship between baseline serum leptin concentrations and weight loss at week 4 ($P = .88$) or at week 24 ($P = .76$).

Body composition changes, as quantified by DXA, are presented in FIGURE 4. Decreases in fat mass showed statistically significant dose responses at 4 weeks and at 24 weeks (Figure 4, top). The loss in fat mass accounted for most of the loss of body mass (more than 95% of the weight loss in the 2 highest-dose cohorts at 24 weeks). Changes in fat-free mass were not significant (Figure 4, bottom).

There was no statistically significant relationship between change in energy intake and dose at week 4 ($P = .87$) or at week 24 ($P = .36$); average (SD) energy intake deficit across all dose groups was 1596 (3788) kJ/d (380 [902] kcal/d) at week 4 and 1819 (3767) kJ/d (433 [897] kcal/d) at week 24. Obese subjects treated with 0.1 and 0.3 mg/kg of rL had lower mean (SD) energy intake than subjects treated with placebo at week 4 (76 [19.7] kJ/kg per day [18.1

[4.7] kcal/kg per day] [n = 25] vs 100 [61.7] kJ/kg per day [23.8 [14.7] kcal/kg per day] [n = 11]), respectively, and at week 24 (85.3 [20.2] kJ/kg per day [20.3] [4.8] kcal/kg per day] [n = 20] vs 101.6 [18.5] kJ/kg per day [24.2 [4.4] kcal/kg per day] [n = 6], respectively) ($P$ = .09 for both comparisons).

### Safety Measures

Injection site reactions mild (86%) to moderate (14%) in severity were the most common adverse events are reported and summarized in TABLE 4. Injection site reactions were generally well tolerated by most subjects; 2 subjects withdrew because of them (Figure 1). For obese subjects who experienced injection site erythema, pruritus, or inflammation (considered characteristic of subcutaneous administration of a protein), the mean (SD) number of such events per subject was 1.7 (0.6), placebo; 3.0 (2.4), 0.01 dose; 3.5 (3.5), 0.03 dose; 27.5 (32.9), 1.0 dose; and 41.6 (57.8), 0.3 mg/kg per day cohorts over the 24-week course of the study.

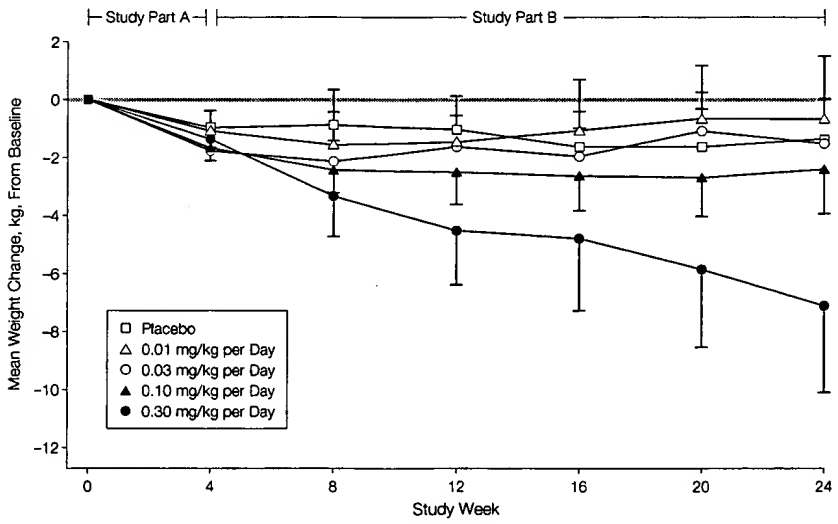
The next most common adverse event was headache, which occurred in 38% and 44% of the placebo- and rL-treated subjects, respectively. None of the subjects taking rL experienced clinically significant adverse effects on major organ systems (central nervous system, cardiovascular, hepatic, renal, gastrointestinal, hematologic) as evidenced by adverse event incidence, physical examinations, laboratory values, electrocardiograms, and vital signs. There were no effects of rL on glycemic control or insulin action, as evidenced by serum insulin and glucose profiles obtained during OGTTs. There were no clinically or statistically significant treatment effects on serum concentrations of luteinizing hormone, follicle-stimulating hormone, cortisol, or prolactin.

### Pharmacokinetics and Antibodies to rL

Maximum serum concentrations of leptin (endogenous leptin plus rL) increased with dose (TABLE 5). Pharmacokinetic analysis demonstrated that serum concentrations of rL peaked approximately 4 hours after injection.[21]

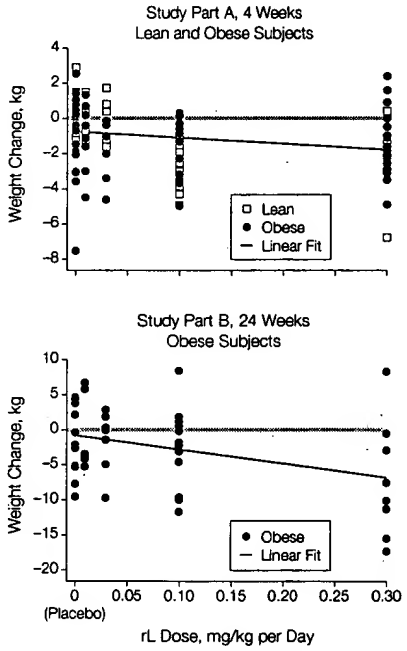
Among subjects from data were available, 32 (38%) of 85 in the 4-week cohort receiving subcutaneous doses and 25 (71%) of 35 in the 24-week cohort tested positive for antileptin antibodies. A statistically significant increasing proportion of subjects were positive for antileptin antibodies with increasing dose (trend test, $P<.01$ and $P<.001$ at 4 and 24 weeks, respectively). Antibody status (positive or negative for the presence of antileptin antibodies) had no statistically significant independent effect on weight loss at 4 weeks ($P$ = .77) or at 24 weeks ($P$ = .12) after accounting for the effects of treatment and dose cohort on weight loss. At 4 weeks, there was no association of the occurrence of adverse events ($P$ = .11) or ISRs ($P$ = .13) with antibody status. By 24 weeks, all subjects had experienced at least 1 adverse event; thus, an association between the overall incidence of adverse events and antibody status could not be determined. There was an association of the occurrence of ISRs ($P$ = .008)

**Figure 2.** Pattern of Weight Change From Baseline to Week 24 in Obese Subjects Who Received Recombinant Methionyl Human Leptin (rL)




Error bars indicate SEM; gray line indicates baseline. The number of subjects is not constant over the course of the study (see Table 2).

**Figure 3.** Relationship Between Recombinant Methionyl Human Leptin (rL) Dose and Body Weight as Measured by Calibrated Scales




$P$ = .02 in study part A, and $P$ = .01 in study part B. Gray line indicates baseline.

with antibody status at 24 weeks: 16 (64%) of 25 antibody-positive subjects experienced ISRs, while 5 (23%) of 22 antibody-negative subjects experienced ISRs.

## COMMENT

These data show that a dose-response relationship exists both after 4 weeks of exposure to recombinant leptin in lean and obese subjects and after 24 weeks of exposure in obese subjects. There was considerable variability in the amount of weight lost by individual subjects; on average, weight loss increased with rL dose. Weight loss in subjects treated with rL was primarily due to fat loss, which accounted for more than 95% of the weight lost among obese subjects in the 2 highest-dosing cohorts after 24 weeks.

These findings do not suggest an absolute leptin resistance in obese individuals with elevated endogenous leptin levels; however, there still may be relative leptin resistance with increasing adiposity. Higher doses of exogenous leptin may be required to provide a sufficient signal for weight loss in subjects with greater adiposity.

We presume that weight loss is related to increased central nervous system exposure to exogenous leptin. In a separate substudy in a group of subjects treated with rL by continuous subcutaneous infusion, CSF leptin concentrations were elevated by exogenous subcutaneous leptin administration.[22] Direct central nervous system administration of rL also induces weight loss in animals.[23-25] These observations are consistent with the hypothesis that the effects of rL on weight are centrally mediated.

The therapeutic potential for rL to treat obesity cannot be determined from the results of this study. Although statistically significant dose-response relationships for weight loss and fat loss were observed in this study, differences between dose groups were not detectable given the study design.

Two children with genetic leptin deficiency have been reported[5]; 1 has been treated with rL and has shown substantial weight loss with a low dose, demonstrating the biologic activity of rL.[26] Three consanguineous people have been found to have very high serum leptin concentrations and a defect in the leptin receptor[27] and would be predicted not to respond to exogenous administration of rL.

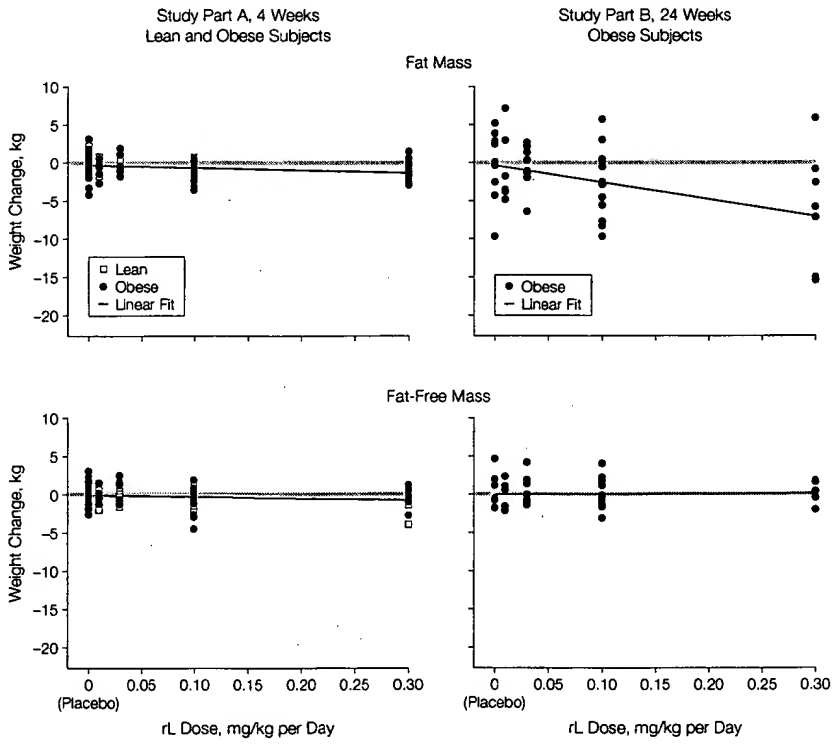
As part of the routine dietary intervention in our study, obese subjects were prescribed a 2100-kJ (500-kcal) deficit diet that, if followed, would lead to an average weight loss of 0.5 kg/wk. Placebo-treated obese subjects lost an average of 1.7 kg in 24 weeks; thus, long-term dietary compliance was poor in these subjects. The actual effect of the dietary intervention was therefore minimal in this study. Lower energy intake

**Table 3.** Comparison of Observed and Imputed Weight Loss Results in Obese Subjects From Baseline to Week 24*

| | Mean (SD) Weight Change From Baseline, kg | | | |
|---|---|---|---|---|
| | Observed | | Imputed | |
| rL Dosage, mg/kg per Day | No. of Subjects | Observed Value | No. of Subjects | LOCF Value |
| Placebo | 12 | −1.3 (4.9) | 20 | −1.0 (3.8) |
| 0.01 | 6 | −0.7 (5.4) | 8 | −0.7 (4.6) |
| 0.03 | 8 | −1.4 (4.1) | 8 | −1.4 (4.1) |
| 0.10 | 13 | −2.4 (5.5) | 16 | −2.1 (5.0) |
| 0.30 | 8 | −7.1 (8.5) | 18 | −3.3 (6.7) |

*LOCF indicates last observation carried forward; rL, recombinant methionyl human leptin.

**Figure 4.** Relationships Between Recombinant Methionyl Human Leptin (rL) Dose and Body Composition (Fat Mass)




In study part A among the lean and obese subjects, $P = .002$ for the change in fat mass and $P = .11$ for fat-free mass. In study part B, $P = .004$ for the change in fat mass and $P = .8$ for fat-free mass. Fat mass was measured by dual-energy x-ray absorptiometry. Gray line indicates baseline.

was reported by subjects treated at the highest doses of rL, suggesting that the weight loss effect may be due to a reduction in food consumption. However, the instrument used (48-hour dietary recall) is relatively insensitive, and there was considerable variability in the reduction of energy intake.

Considered a characteristic of the injection technique, injection site ecchymosis was the most common adverse effect and occurred among 71% of those treated with placebo and 62% of those treated with rL. Symptoms considered characteristic of subcutaneous administration of a protein, such as injection site erythema, pruritus, and inflammation, occurred with greater incidence in rL-treated subjects than in placebo-treated subjects. Injection site reactions did not appear to unblind the study as they were not unique to the subjects receiving rL. Injection site reactions were generally treated with topical creams and antihistamines, most resolved over a few weeks, and they did not appear to contribute significantly to the dropout rates.

Antibodies to leptin were observed in 38% and 71% of rL-treated subjects at 4 and 24 weeks, respectively; however, these antibody levels had no relationship with weight loss or overall adverse event incidence. At 24 weeks, antibody-positive subjects had a higher incidence of ISRs; however, the incidence of both ISRs and seroreactivity increased with dose, so a causal relationship of seroreactivity to dose should not be inferred. In subjects who tested positive for antibody formation, higher rL serum concentrations were observed on day 28 compared with day 1 and day 14 results.[21] These data are consistent with pharmacokinetic data obtained in animals, which showed that following formation of antibodies against rL, serum concentrations of rL increased.[28]

With the exception of 2 subjects who withdrew for ISRs and 1 who withdrew for palpitations, dropouts did not appear related to adverse events, and adverse events (other than ISRs) did not appear related to the rL dose. Subject withdrawals were greatest in the highest-dose cohort. The majority of withdrawals among obese subjects was between the initial 4-week part A and the 20-week part B study. We believe that injection volume or number of injections influenced the decision to withdraw. Sensitivity analyses using imputed values for subjects who withdrew led to similar conclusions about the dose-response relationship of rL treatment to weight loss.

Researchers have expressed some concern that leptin may exacerbate insulin resistance or contribute to type 2 diabetes.[29] Based on OGTT performed at baseline and after 4 and 24 weeks of treatment with rL, we found no indication that exogenous leptin affected glycemic control. Because these subjects were selected based on expected normal glucose tolerance, it was not possible to document an improvement in abnormal glycemic control associated with treatment.

In conclusion, these results demonstrate that subcutaneous bolus injections of rL result in weight loss in some individuals, and show that the weight loss caused by rL may be due almost

**Table 4.** Subject Incidence of Injection Site Reactions (ISRs) for Obese Subjects

| | Subject Incidence of ISRs* | | | | |
|---|---|---|---|---|---|
| | | rL Dosage, mg/kg per Day | | | |
| | Placebo (n = 21) | 0.01 (n = 8) | 0.03 (n = 8) | 0.10 (n = 18) | 0.30 (n = 18) |
| Any ISR | 16 (76) | 7 (88) | 5 (63) | 12 (67) | 10 (56) |
| Injection site | | | | | |
| Ecchymosis | 15 (71) | 6 (75) | 5 (63) | 11 (61) | 10 (56) |
| Erythema | 2 (10) | 4 (50) | 2 (25) | 10 (56) | 10 (56) |
| Pruritus | 2 (10) | 1 (13) | 0 (0) | 6 (33) | 5 (28) |
| Inflammation (induration) | 1 (5) | 0 (0) | 0 (0) | 5 (28) | 4 (22) |
| Pain | 4 (19) | 0 (0) | 0 (0) | 2 (11) | 4 (22) |
| Rash | 0 (0) | 3 (38) | 0 (0) | 1 (6) | 5 (28) |
| Reaction† | 1 (5) | 0 (0) | 0 (0) | 1 (6) | 4 (22) |
| Edema | 0 (0) | 0 (0) | 0 (0) | 4 (22) | 0 (0) |

*Subject incidence was defined as the number of subjects who experienced the indicated event at least once at any time during both parts of the study. rL indicates recombinant methionyl human leptin. All data are number (percentage).
†This term was used to capture other symptoms such as warm skin, dry skin, and flaky skin.

**Table 5.** Maximum Serum Leptin Concentrations*

| | Maximum Serum Leptin Concentration, ng/mL† | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | rL Dosage, mg/kg per Day | | | | | | | |
| | No. of Subjects | Placebo | No. of Subjects | 0.01 | No. of Subjects | 0.03 | No. of Subjects | 0.10 | No. of Subjects | 0.30 |
| Baseline | 37 | 15.9 (19.7) | 16 | 16.8 (17.8) | 16 | 16.4 (16.1) | 31 | 12.3 (12.3) | 26 | 15.1 (12.7) |
| Day 1 | 38 | 20.9 (22.4) | 16 | 30.3 (26.4) | 16 | 53.6 (31.1) | 31 | 131.5 (34.3) | 26 | 357.6 (105.3) |
| Week 4 | 35 | 15.3 (17.1) | 15 | 22.9 (17.6) | 16 | 57.6 (28.9) | 28 | 170.9 (135.3) | 26 | 667.0 (555.7) |
| Week 24‡ | 12 | 25.0 (39.6) | 6 | 28.3 (20.2) | 6 | 115.5 (99.9) | 13 | 271.7 (322.4) | 8 | 480.3 (522.0) |

*All data are presented as mean (SD).
†Concentrations reported herein are the maximum value following the dose on the indicated days. Note that the assay did not distinguish between endogenous leptin and recombinant methionyl human leptin (rL).
‡Obese subjects only.

entirely to fat loss. These results also suggest that rL has an acceptable short-term (≤6 months) safety profile. Although additional human studies of leptin analogs are necessary to determine its therapeutic potential for the treatment of obesity and diabetes, many questions remain about the possibility of using a leptin receptor agonist as a therapeutic agent to treat obesity. Our findings show, however, that some patients, across a wide range of body weights, respond to exogenous leptin administration.

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EXHIBIT B

# The endocrine secretion of human insulin and growth hormone by exocrine glands of the gastrointestinal tract

**Ira D. Goldfine*, Michael S. German, Hsien-Chen Tseng, Juemin Wang, Janice L. Bolaffi, Je-Wei Chen, David C. Olson, and Stephen S. Rothman**

*Schools of Medicine and Dentistry, University of California at San Francisco, San Francisco, CA, and the Genteric Corporation, Berkeley, CA.*
**Corresponding author (e-mail: idg@itsa.ucsf.edu).*

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**The exocrine pancreas, liver, and submandibular glands of the rat were used to express and secrete two exogenous, human protein hormones (growth hormone and insulin) into blood at physiological concentrations. Transfection, expression, and secretion were achieved by the in vivo retrograde injection of plasmid DNA into the secretory ducts of these glands. Pancreatic acinar cells secreted physiological concentrations of growth hormone into the circulation, and its secretion was enhanced by cholinergic stimulation. A human insulin gene was engineered to allow normal processing of insulin in non-β cells. With this gene, the secretion of human insulin by the exocrine pancreas normalized elevated blood glucose levels in diabetic rats. These in vivo observations demonstrate the utility of retrograde ductal administration of naked DNA into exocrine organs as a novel method for the regulated systemic delivery of protein-based pharmaceuticals.**

Keywords: gene therapy, diabetes, protein delivery

The ability to replace defective or absent genes has attracted wide attention as a method to treat human diseases and disorders[1-3]. Gene-based therapy may also be useful as a method for the systemic administration of protein pharmaceuticals. Such an approach would be extremely beneficial for the treatment of a wide range of disorders that require the administration of circulating proteins including hormones, growth factors, clotting proteins, and other polypeptides[4-6]. The ultimate success of such a procedure depends upon the development of effective in vivo methods to manufacture the desired protein and secrete it into blood[7,8].

For a variety of reasons, the exocrine glands of the gastrointestinal system, especially the pancreas, liver, and major salivary glands, are particularly well suited for this purpose. First, these glands are major protein-synthesizing and protein-secreting systems. For example, the human exocrine pancreas manufactures and secretes approximately 20 g of protein daily. Second, like the parenchymal cells of the liver, the exocrine cells of the salivary glands and pancreas secrete substantial amounts of protein into the blood[9-11]. Third, in vivo, these three glands can be accessed noninvasively by the retrograde administration of DNA vectors directly into their duct systems. For example, in conscious humans, the vector can be administered either by cannulation of a collecting duct of a major salivary gland in the mouth or by cannulation of the common bile duct via endoscopic retrograde cholangiopancreatography. Fourth, the cells of these glands form a monolayer that encloses the duct system, and as a consequence, all secretory cells can be accessed by a single injection of a vector. This approach avoids both dilution of the vector and the need for tissue targeting. Fifth, because the vector is presented to the cells from outside the body, the immunological and inflammatory responses elicited when a vector is administered into blood may be largely avoided. Sixth, protein secretion by the salivary glands and pancreas is regulated by a variety of hormones and neurotransmitters that are natural components of the feeding response, permitting both regulated secretion and automatic dosing with feeding[12-14].

The current studies were undertaken to demonstrate the feasibility of using these glands for the treatment of human disease by establishing that they could be transfected via their duct systems, express engineered proteins, and then secrete these proteins into the circulation. To achieve these goals, we prepared plasmid DNA for human insulin and growth hormone (hGH), and injected them into the rat pancreas, liver, and submandibular glands by retrograde perfusion of their secretory ducts. The hormones subsequently were expressed in these tissues and secreted into blood. The data indicate that transfection of the exocrine glands of the gastrointestinal tract can be an effective and clinically advantageous method for the systemic delivery of therapeutic proteins.

## Results

**Expression and secretion of hGH.** Several plasmid configurations were tested in vivo to determine optimal promoter sequences. Forty-eight hours after plasmids containing the coding sequence for hGH were injected into the pancreatic duct, the hormone was measured in pancreatic tissue (Fig. 1A). Among the promoters tested, the cytamegalovirus (CMV) promoter was by far the most effective and produced high levels of hGH in tissue (in the range of 150 ng/g tissue wet weight) when compared with either promoterless controls, or plasmids containing Rous sarcoma virus (RSV) and chymotrypsin promoters (Fig. 1A). A cationic lipid adjuvant (Lipofectin) increased expression by 50%, and premixing the plasmid with adenovirus enhanced tissue expression fivefold (Fig. 1B). Expression of hGH at 24, 48, or 72 hours after injection was similar under all conditions studied (data not shown).

After transfection, hGH was secreted into plasma (Fig. 1C and D). Plasmids containing the CMV promoter increased circulating levels of hGH five times above background (Fig. 1C). With plasmid alone, plasma concentrations in the range of 60–80 pg/ml were routinely observed. Premixing the plasmids with adjuvants also increased circulating hGH levels (Fig. 1D). Lipofectin increased

plasma levels by an additional 50%, and adenovirus by 75%, when compared to plasmid alone.

To identify the pancreatic cells that expressed the recombinant protein, we localized a marker protein, green fluorescent protein (GFP) by immunohistochemistry. Tissue samples were obtained 72 hours after the GFP plasmid was injected into the pancreatic duct. Staining for GFP was observed in the pancreas of animals treated with GFP DNA, but not in untreated animals (Fig. 2). GFP expression was restricted to exocrine cells; there was no staining in either ductal or islet cells. Expression was observed in 0.1–1.0% of these cells. Under the study conditions there was no histological indication of inflammatory infiltration as a consequence of ductal injection of the vector.

To further explore the utility of the method, we measured hGH expression in liver after injection into the hepatic duct of an hGH plasmid premixed with adenovirus. Again, hGH was expressed in tissue and detected in plasma. Levels in liver were far lower than in the pancreas (< 1 ng/g, as compared to about 500 ng/g), but hGH concentration in plasma was nonetheless comparable (in the range of 0.15 ng/ml; Fig. 3). When both pancreas and liver were transfected simultaneously through the pancreatic-biliary tree in the same animals, plasma levels were higher than when the glands were treated individually (nearly 0.3 ng/ml, a value nearly equal to the sum of that observed for the two organs separately).

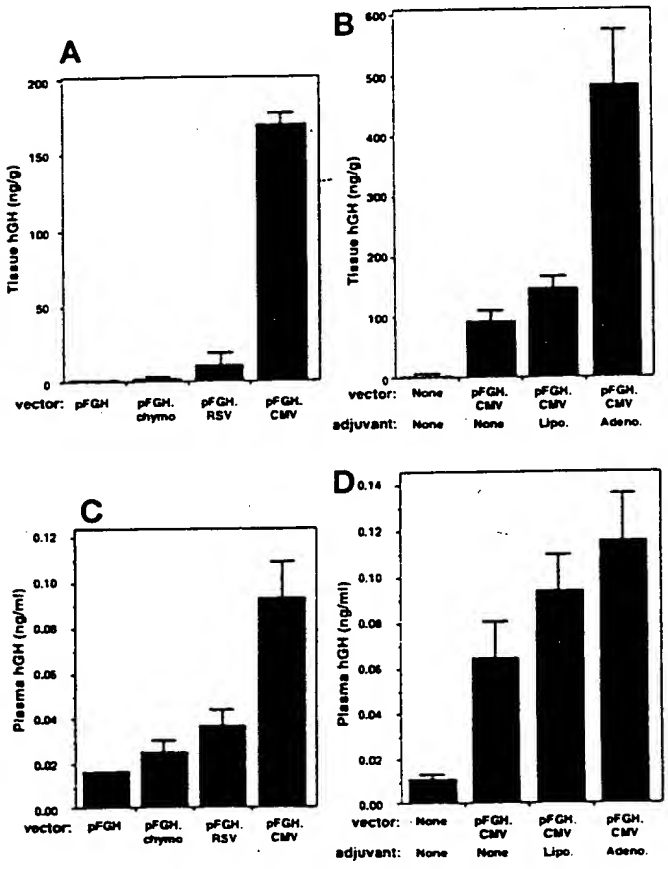


Figure 1. Human growth hormone (hGH) expression in rat exocrine pancreas and plasma. (A) Effect of various promoters on hGH expression (plus Lipofectin; n = 2,4,5,13). (B) Effect of adjuvants on hGH expression (n = 5,10,13,16). (C) Effect of various promoters on hGH secretion (plus Lipofectin; n = 2,4,5,27). (D) Effect of adjuvants on hGH secretion (n = 4,10,27,14). In all figures the data shown are the mean ± the SEM. pFGH = promoterless plasmid; pFGH.chymo = plasmid with the chymotrypsin promoter; pFGH.RSV = plasmid with the Rous sarcoma virus promoter; pFGH.CMV = plasmid with the cytomegalic virus promoter; Lipo. = Lipofectin; Adeno. = adenovirus.

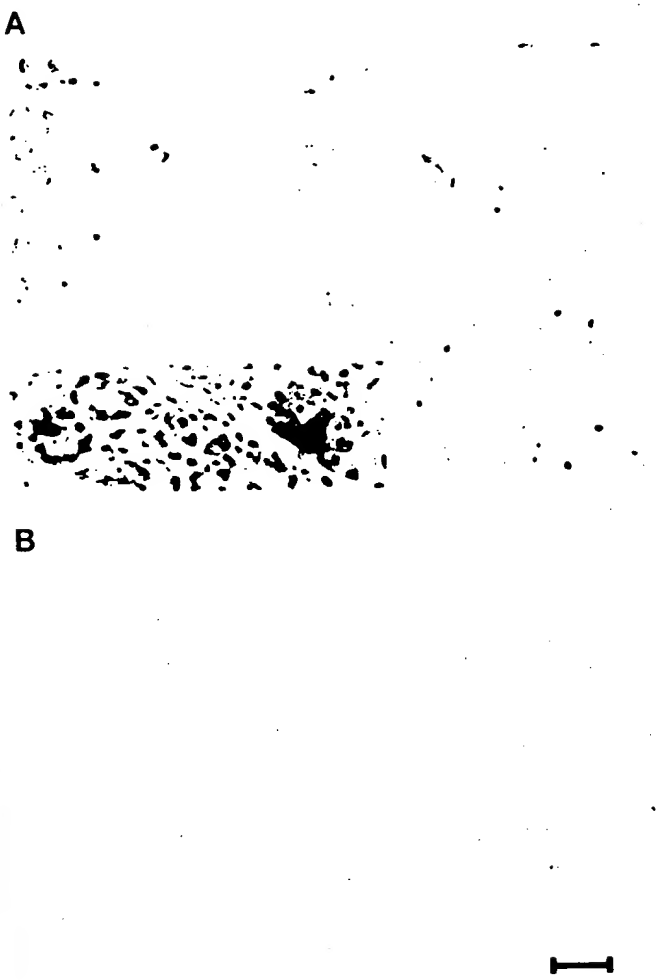


Figure 2. Detection of GFP marker protein in acini of the exocrine pancreas. GFP protein was detected in acinar tissue (reddish-brown stain) by immunohistochemistry (top and inset). No stain was detected in an adjacent control section (bottom). Bar indicates 60 microns in the inset, and 273 microns in the top and bottom panels.

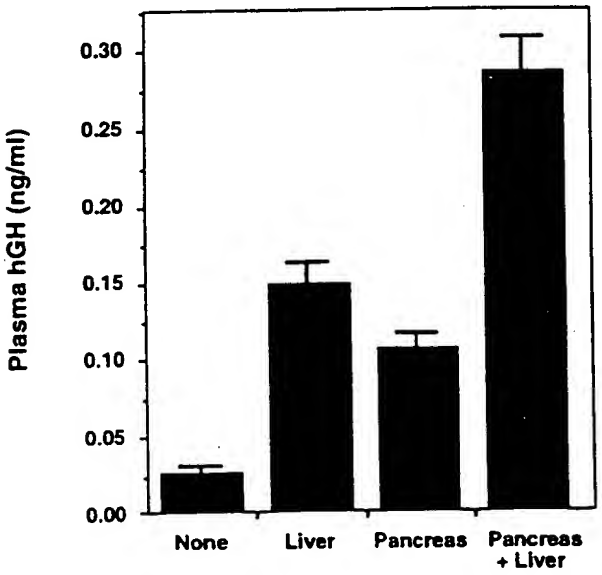


Figure 3. Comparison of hGH secretion by rat liver, pancreas, and combined liver and pancreas. Left to right, n = 4,6,14,3.

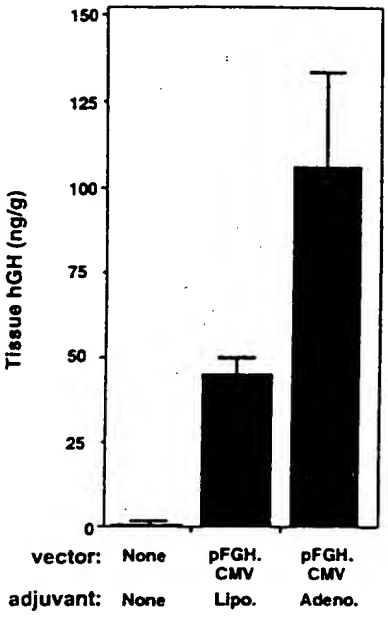


Figure 4. hGH expression in rat salivary gland. Left to right, n = 5,4,5.

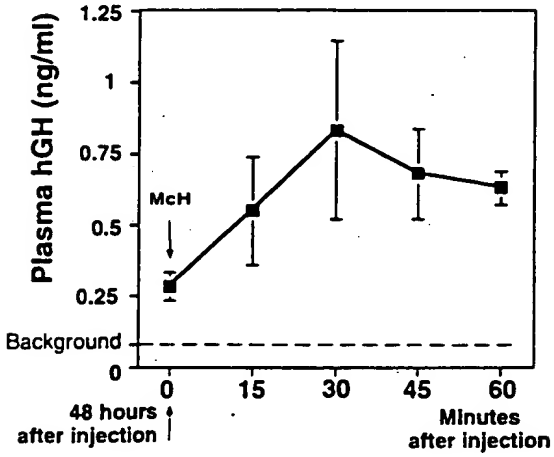


Figure 5. Stimulation of hGH secretion by a cholinergic agonist (McH). Left to right, n = 4.

Experiments were also carried out using submandibular glands. Forty-eight hours after the retrograde injection of an hGH plasmid (with Lipofectin) into Wharton's duct, tissue levels of hGH averaged about 50 ng/g tissue wet weight (Fig. 4), and plasma hGH levels were in the 20–40 pg/ml range. As in the pancreas, the addition of adenovirus increased tissue hGH levels, in this case to 100 ng/g (Fig. 4).

**Regulation of hGH secretion.** To determine whether secretion of the engineered protein would be enhanced during feeding, pancreatic secretion was augmented with a secretory stimulant. For animals in these experiments, both pancreas and liver were transfected. Two days after transfection, the rats were treated with the cholinergic agonist, acetyl-β-methylcholine (McH). Secretion of hGH was increased threefold within 30 minutes of stimulation, with plasma levels approaching 1.0 ng/ml (Fig. 5). Similar enhancement of hGH secretion was observed when the pancreas was studied alone, and when the salivary glands were studied alone (data not shown).

**Insulin and diabetes.** To use this technique to treat a disease state (diabetes mellitus) we expressed human insulin in the exocrine pancreas. Diabetes was induced in rats by administration of the β-cell toxin, streptozotocin. As a consequence, blood glucose levels rose from the normal level of 100 mg/dl to 300–400 mg/dl within 24 hours and remained elevated (Fig. 6A). Immunohistochemical examination of streptozotocin-treated animals revealed widespread damage to islets, with few remaining β cells (data not shown). One hour after streptozotocin administration, the pancreas was transfected by retrograde ductal injection of a plasmid containing the coding sequence for human insulin. The insulin cDNA was modified to allow for correct proteolytic processing by non–β-cells. Treatment with the human insulin plasmid reduced blood glucose levels in diabetic rats to the normal range (Fig. 6A and C), and concentrations of insulin remained near pretreatment values (Fig. 6B). Blood glucose levels in the insulin–gene treated animals remained euglycemic for 7 days, whereas animals transfected with a control plasmid were diabetic (Fig. 6C).

## Discussion

We used the exocrine pancreas, liver, and submandibular glands of the rat to manufacture and secrete engineered human hGH and insulin into the circulation. Using DNA incorporated into viral particles, several groups have previously reported expression of human proteins in salivary glands, liver, and pancreas[1–21]. We have now been able to achieve positive results with either naked DNA or DNA premixed with adjuvants, by the retrograde injection of

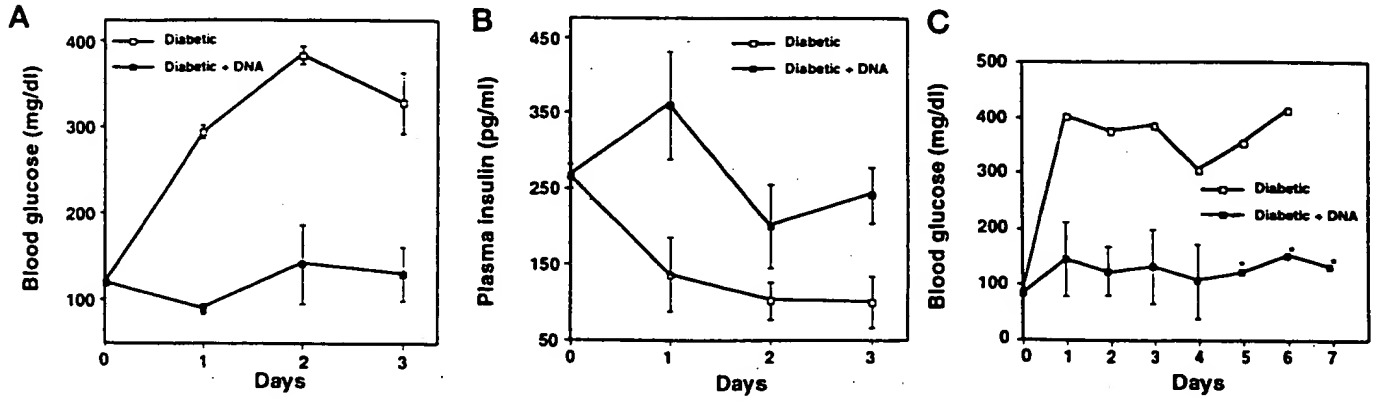


Figure 6. Human insulin expression and secretion in diabetic rat pancreas. (A) Plasma glucose levels in diabetic rats, and diabetic rats treated with the pBAT16.hInsG1.M2 plasmid (n = 3). Values are the mean ± SEM. The changes in glucose values were significantly different ($p < 0.001$). (B) Plasma insulin levels in diabetic rats, and diabetic rats treated with the pBAT16.hInsG1.M2 plasmid (n = 3). The changes in insulin values were significantly different ($p < 0.001$, $< 0.05$, and $< 0.01$). (C) Plasma glucose levels in pBAT16.hInsG1.M2 plasmid-treated diabetic rats, measured over 7 days (n = 3, days 1–4; *n = 2, days 5–7).

human DNA into the duct system of these organs. It was not necessary to incorporate the DNA into viral particles. The efficacy of naked DNA was unexpected, because in tissue culture it is not normally taken up by cells without special permeabilization procedures. How DNA enters the secretory cells from the duct system in vivo is unknown, but a recent study suggests one possible mechanism. Using an atomic force microscope, large pores of 150 nm diameter have been identified in apical membranes of acinar cells of the exocrine pancreas[25]. Such pores are sufficiently large for 5 nm diameter plasmids to penetrate by diffusion. This observation is consistent with earlier studies, which demonstrated that the apical membrane of the pancreatic acinar cell is permeable to large, polar nonelectrolytes including mannitol, sucrose, and inulin[26,27].

We found that rapid regulation of blood levels of engineered proteins (e.g., peptide hormones) can be achieved by using exocrine cells. Exocrine secretory cells store large amounts of protein, but under unstimulated conditions protein secretion is limited to a low rate. Greater rates of secretion are achieved rapidly in response to a variety of stimulants. We observed that the secretion of hGH into the circulation from the exocrine pancreas and salivary gland was increased by the administration of a cholinergic agonist. In comparison, hepatocytes do not store large amounts of protein, but secrete what they manufacture soon after synthesis. This difference between exocrine and hepatic cells was reflected in how they handled hGH. Although both glands yielded similar levels of hGH in plasma after the same transfection regimen, the concentration of hGH in tissue was 500-fold lower in liver than in exocrine pancreas. For this reason, the liver can serve as a source of basal or constitutive secretion of engineered proteins, whereas the pancreas can serve as a source for their regulated secretion. Moreover, it is not necessary to administer exogenous stimulants to elicit regulated secretion, because secretion is naturally elevated by the intrinsic physiological mechanisms of feeding. This process is particularly useful for the secretion of engineered proteins (e.g., insulin) that are normally released as part of the digestive process.

In the pancreatic β cell, the secretion of insulin is under the integrated regulatory control of metabolites (such as glucose and amino acids), neurotransmitters (such as acetylcholine), and several peptide hormones (including cholecystokinin). Similarly, secretion from pancreatic exocrine cells during digestion is also stimulated by some of the same neurotransmitters and hormones (in particular, acetylcholine and cholecystokinin). Moreover, like the β cells, exocrine cells are also responsive to changes in the concentration of digestive metabolites, such as glucose and amino acids[28]. The presence of these common regulatory pathways raises the possibility that the secretion of engineered insulin from pancreatic and perhaps other exocrine cells could occur in a manner similar to that of β cells, and thus regulate glucose homeostasis in a normal manner. In experiments designed to test this possibility, rat pancreatic exocrine cells were transfected to produce and secrete human insulin in rats made diabetic with streptozotocin. Not only did the insulin secreted from the exocrine cells lower the elevated blood glucose levels, it also produced and maintained near-normal glucose homeostasis. Although the detailed mechanisms that underlie this regulation are unknown, the data indicate that insulin secretion by exocrine cells may be regulated in a fashion similar to that of β cells.

## Experimental protocol

**Animal preparations.** Male Sprague-Dawley rats, 260–280 g, were fed a balanced meal of lab chow at all times except when fasted overnight (water ad lib). Animals were anesthetized with pentobarbital (50 mg/kg body weight) and the following procedures were carried out. For the pancreas, the abdominal cavity was opened and the pancreatic duct cannulated external to the duodenum with polyethylene (PE) 10 tubing. The biliary duct was occluded with a temporary ligature, and then the appropriate DNA construct (8–25 μg DNA in 100 μl of phosphate buffered saline (PBS)) was injected retrograde into the duct system. Dose-response studies indicated that maximal response was obtained with 8–25 μg DNA, with more DNA resulting in reduced activity (data not shown). In studies using Lipofectin (LTI/BRL; Life Technologies, Gaithersberg, MD) (v.i.), it was added to a final concentration of 6–12% v:v with DNA. When adenovirus was added (v.i.), $3 \times 10^{\ }$ viral particles were mixed with the plasmid immediately prior to injection. The material was kept in the duct for 5 min prior to establishing normal flow. The abdomen was then closed and the animals allowed to recover. Cholinergic stimulation was performed by injection of acetyl-β-methylcholine (0.8 mg/kg body weight) 48 h after administration of DNA. For the submandibular glands, both the left and right Wharton's duct, were cannulated intraorally with PE 10 tubing. The DNA construct was injected into the duct system of each gland in a retrograde fashion (4 μg/50 μl of PBS). The material was kept in place for 2 min before normal flow was reestablished. For the liver, the biliary duct was cannulated with PE 10 tubing external to the duodenum. The tubing was advanced to the bifurcation of the hepatic duct in order to prevent injected material from entering the distally located pancreatic drainage. The appropriate DNA construct (8 μg DNA in 100 μl of PBS) was injected retrograde into the duct and kept in place for 2 min prior to establishing normal flow. In some experiments both the liver and pancreas were treated by retrograde injection. In this case, injection was made into the hepatic duct first, and then the tubing was partially withdrawn to provide access to the pancreatic duct system. A temporary ligature was then placed around the hepatic duct to prevent the second infusion from entering the parenchyma of the liver.

**Sampling tissue and blood.** The animals were anesthetized again at the times specified. In general, blood was drawn in late morning after 15 h of fasting. In some experiments a sample of blood was taken (either from the femoral vein or inferior vena cava), and the transfected tissue was then removed. In other studies, a cholinergic agonist was administered subsequent to blood sampling, and additional blood samples were taken at various time points. Subsequently, the tissue was removed and homogenized in PBS containing 5 mM $Na_2HPO_4$ (pH 7.8) at a tissue to fluid ratio of 1:10 using a motorized mortar and pestle. Large particulate material in the homogenate was removed by sedimentation at 10,000 × g for 30 min, and the supernatant was assayed for the protein of interest. Streptozotocin (65 mg/kg of body weight, in 1 mM citrate buffer, pH 4.5; Sigma, St. Louis, MO) was given to fasted animals by intraperitoneal injection 1 h before introduction of the gene vector.

**Immunohistochemistry.** Samples were fixed in 5% buffered formalin for 24–48 h at room temperature. Fixed tissues were dehydrated and embedded in paraffin, and 5 μm sections were made using standard techniques. Prior to antibody hybridization, endogenous peroxidase was quenched in 0.7% $H_2O_2$/MeOH, and antigen retrieval was performed using Citra solution (Biogenex, San Ramon, CA) according to the manufacturer's instructions. Sections were preincubated for 30 min in 5% goat serum/PBS, and then incubated overnight at 4°C with primary antisera to GFP diluted 1:1500 in 5% goat serum/PBS (Clonetech, Palo Alto, CA). For negative controls, we used nonspecific rabbit serum (1:1500), stained sections from pancreas injected with a sham plasmid, and omitted the primary antiserum. The following day all sections were incubated with biotinylated goat antirabbit antiserum (5 μg/ml; Vector, Burlingame, CA) for 30 min at room temperature, washed, and then incubated with streptavidin-aminohexanol-biotin HRP complex for 30 min (Vectastain-Elite, Vector). Protein was visualized with the peroxidase substrate 3,3-diamino-benzidine tetrahydrochloride (DAB; Sigma). The color reaction was followed by a brief counterstain in 1% methyl green (Sigma) prior to mounting.

**Vectors and hormone assays.** Four vectors containing hGH were prepared using recombinant DNA methods. Vector pFGH contains the genomic hGH DNA sequence, without promoter, in the pFOX plasmid[29]. pFGH.CMV contains an insertion of the immediate early promoter of human CMV. This vector yields high-level expression in rodent cells. Two similar constructs, pFGH.RSV and pFGH.chymo, that contain hGH under regulatory control of the RSV long term repeat (LTR) promoter or chymotrypsin B gene promoter, respectively, were also used. Human insulin was expressed from the pBAT16.hInsG1.M2 vector plasmid. This vector contains the human insulin cDNA linked to a CMV immediate early promoter, which is positioned upstream of the first intron of human β-globin. The human insulin cDNA was mutated to convert the second protease site, between peptides C and A, to a furin recognition site. This allows for correct proteolytic processing of mature insulin in nonendocrine cells. GFP cDNA from plasmid pEGFP.C2

(Clonetech) was inserted into pFOX. The EGFP sequence was modified to contain a SV40 nuclear localization signal, in frame at the 3' end. This addition allows for partial nuclear localization and facilitates immunohistochemical detection. The CMV immediate early promoter was positioned upstream of the first intron of human β-globin to create the expression vector pFOX.EGFP.N2.CMV. Lipofectin was used according to the manufacturer's instructions at the concentrations indicated. Adenovirus (Ad5-*dl* 342) was mixed with DNA immediately prior to administration in the amounts indicated. Human growth hormone was measured by immunoassay employing a human-specific, coated-bead method (Nichols Institute, San Juan Capistrano, CA), and human insulin was measured by double antibody radioimmunoassay (Linco Laboratories, Saint Louis, MO). The specificity of this latter assay is 100% for human insulin, 38% for human proinsulin, and 60% for rat insulin. Blood glucose was measured by the glucose oxidase method (Lifescan, Milpitas, CA).

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